REMARKS

In view of the above amendments and the following remarks, reconsideration of the outstanding office action is respectfully requested. Pursuant to 37 CFR § 1.121, attached as Appendix A is a Version With Markings to Show Changes Made.

The rejection of claims 1-5, 9-12, 16-20, 23-25, 27-29, 31-36, 40-43, 46-50, 53-62, 70-74, 77-81, and 93-94 under 35 U.S.C. § 112 (1st para.) for failure to meet the written description requirement is respectfully traversed.

As amended, claim 1 is drawn to a DNA construct comprising "a fragment of a trait DNA molecule which has a length that is insufficient to independently impart a desired trait to plants transformed with said fragment of a trait DNA molecule, wherein the fragment of a trait DNA molecule is from a viral source and is at least 110 nucleotides in length but is less than a full-length cDNA encoding said trait" and "a silencer DNA molecule effective to achieve post-transcriptional gene silencing of said fragment of a trait DNA molecule and coupled to said fragment of a trait DNA molecule wherein said trait DNA molecule and said silencer DNA molecule are heterologous to each other and collectively impart the trait to plants transformed with said DNA construct and wherein said fragment of a trait DNA molecule and said silencer DNA molecule are heterologous to plants."

With these limitations describing the claimed "fragment of a trait DNA molecule" and "silencer DNA molecule," applicants submit that one of ordinary skill in the art, having read the present application, would have understood that applicants were in possession of the claimed invention. Therefore, the rejection of claims 1-5, 9-12, 16-20, 23-25, 27-29, 31-36, 40-43, 46-50, 53-62, 70-74, 77-81, and 93-94 under 35 U.S.C. § 112 (1st para.) for failure to meet the written description requirement should be withdrawn.

The rejection of claims 1-5, 9-12, 16-20, 23-25, 27-29, 31-36, 40-43, 46-50, 53-62, 66-74, 77-81, and 93-94 under 35 U.S.C. § 112 (1st para.) for lack of enablement is respectfully traversed.

It is the position of the U.S. Patent and Trademark Office ("PTO") that the present application, while enabling for DNA constructs comprising a trait DNA molecule from the tomato spotted wilt and silencer DNA molecules that are the green fluorescent protein and the turnip mosaic potyvirus genes, a method of using such constructs to impart resistance to turnip mosaic potyvirus and tomato spotted wilt virus to a plant, and plants so transformed, the specification is not enabling for DNA constructs comprising any trait DNA

and any silencer DNA and methods of using them to impart any trait. Applicants respectfully disagree.

As demonstrated below, based on the accompanying Declaration of Dennis Gonsalves Under 37 C.F.R. § 1.132 ("Gonsalves Declaration"), as well as the exhibits referenced therein, the disclosure of the present application would have enabled a skilled scientist to prepare additional DNA constructs having a fragment of a trait DNA from a viral source that has at least 110 nucleotides but is less than a full-length cDNA, and a silencer DNA molecule, and to use such constructs to confer a desired trait (e.g., resistance against viral pathogens) to plants by transforming plants with such constructs.

Applicants submit that data in the present patent application shows that multiple virus resistance can be obtained by transforming plants with a DNA construct that has a silencer DNA (e.g., 726 bp DNA of the green fluorescent protein (GFP) gene, an approximately 400 bp fragment of the nucleoprotein (NP) DNA of tomato spotted wilt virus (TSWV), or the turnip mosaic virus (TurMV) coat protein (CP) DNA) that is linked to short fragments (about 200 bp to 87 bp) of tospovirus (groundnut ringspot virus, impatiens necrotic spot virus, and TSWV) coat protein nucleotide sequence (Example 7) (Gonsalves Declaration ¶ 6). This data also shows that when short DNA segments (200 bp or less) were used in a DNA construct without being linked to a silencer DNA molecule, the construct did not impart resistance to a plant transformed with that construct (Example 5) (Id.). The present application shows that resistance to different viruses can be achieved using the constructs of the present invention, as demonstrated by the resistance imparted to transgenic plants against three tospoviruses, and against a potyvirus and tospovirus (Example 7, with Tables 5-6) (Id.).

Applicant's previous work on developing transgenic papaya for resistance to papaya ringspot virus (PRSV) showed that some strains of PRSV could overcome the resistance in some transgenic papaya lines (Gonsalves Declaration ¶ 7). In particular, the transgenic 'Rainbow' papaya is commercially grown in Hawaii and virtually saved the papaya industry from destruction by PRSV (Id.). However, inoculation experiments have shown that 'Rainbow' is resistant to PRSV strains in Hawaii but is susceptible to a number of PRSV strains from outside of Hawaii (Id.). This differential resistance is largely due to the differences in nucleotide homology between the coat protein genes of different PRSV strains (Id.). In Hawaii, the PRSV strains share at least 97% homology to the PRSV coat protein transgene of 'Rainbow' (Id.). However, some strains, for example, YK from Taiwan and TH from Thailand, share only 89-90% homology to the CP transgene of 'Rainbow' (Id.). Thus,

strains of PRSV from Taiwan or Thailand could cause severe damage to the Hawaiian papaya industry if they were introduced to Hawaii (Id.).

Recently, it was also reported from Okinawa that another potyvirus, papaya leaf distortion mosaic virus (PLDMV), causes symptoms similar to PRSV on papaya but is not related to PRSV (Gonsalves Declaration ¶ 8). In fact, the coat protein of PLDMV shares only 49-59% amino acid similarity to the coat protein of PRSV (Id.). Greenhouse inoculations also showed that 'Rainbow' is susceptible to PLDMV. As with various PRSV strains, PLDMV could cause severe damage to the Hawaiian papaya industry if it was introduced to Hawaii (Id.).

In order to develop a Hawaiian transgenic papaya that would be resistant to outside strains of PRSV and simultaneously to PLDMV, DNA constructs in accordance with the present invention were prepared (Gonsalves Declaration ¶ 9). These DNA constructs had a trait DNA that was less than a full-length trait-encoding DNA but containing at least 110 nucleotides, coupled to a DNA silencer molecule, with both the trait and the DNA silencer molecules under the control of a single 35S promoter and single terminator sequence (Id.). Various coding sequences for segments of the PRSV or PLDMV coat protein were incorporated into the DNA constructs (Id.). The constructs were then cloned into a suitable plant expression vector (Id.). Papaya plants were then transformed with the expression vectors containing the DNA constructs and the transgenic papaya were analyzed for resistance to the target viruses (Id.). The data from this work, described in detail herein *infra*, shows the effectiveness of the DNA constructs of the instant invention in conferring multiple viral resistance to plants transformed with such constructs (Id.).

Using the techniques described in Example 1 of the present application, basic gene constructs containing the green fluorescent protein (GFP) or one-half of the DNA molecule encoding the nucleocapsid protein (1/2NP) of TSWV under the control of a single promoter and terminator sequence, (i.e., [Promoter]-[GFP]-[DNA segments]-[Terminator] and [Promoter]-[1/2NP] [DNA segments]-[Terminator], respectively) were prepared (Gonsalves Declaration ¶ 10). Various trait DNA fragments from a variety of PRSV strains, and/or PLDMV DNA segments, in translatable or non-translatable configurations, and a DNA useful for antibiotic transgene selection following transformation, were inserted into these DNA constructs (Id.).

Immature zygotic embryos extracted from seeds of immature green 'Sunrise' or 'Kapoho' papaya were transformed with expression vectors containing the DNA

constructs prepared as described above and then transformed (Gonsalves Declaration ¶ 11). Transgenic embryos were regenerated in a medium containing the appropriate antibiotic for selection (Id.). Mature somatic embryos surviving selection were transferred to germination medium and allowed to develop into plantlets with dark green leaves and root initials (Id.). Plants were placed in rooting medium and transferred to the greenhouse (Id.). Transgenic lines from the germination medium were analyzed by PCR to confirm that the viral DNA was in the plantlets (Id.). Northern blots were carried out to detect the level of RNA expressed in transgenic lines, and the copy number of the transgene in the transgenic plants was determined by Southern blot analysis (Id.). Transgenic plants were further cultivated in accordance with conventional procedures so that the DNA construct was present in the resulting plants (Id.). To test viral resistance, plant inoculations with virus were carried out as previously described and systemic symptoms of infection were recorded (Id.).

The results are summarized in Table 1 and Table 2 (attached to the Gonsalves Declaration as Exhibits 6 and 7, respectively) (Gonsalves Declaration ¶ 12). The data from Table 1 show resistance against the PRSV strain from Keaau, Hawaii (KE), which is the first virus that the RO plants were tested against (Id.). The data clearly show that a segment of KE DNA (~ 200 nucleotides in length) imparts resistance when linked to the silencer DNA (Id.). This demonstrates that the present patent application teaches constructs and a method for conferring resistance to additional viral plant pathogens such as PRSV (Id.).

Plants that were resistant to KE were then screened for resistance to the other target viruses for which segments of the CP were present in the transgenic papaya (Table 2) (Exhibit 7) (Gonsalves Declaration ¶ 13). In particular, plants were first screened for resistance to KE and, subsequently, the KE-resistant plants were screened for resistance to the other strains of PRSV or to PLDMV (Id.). The data in Table 2 show that applicant's approach provides resistance to various strains of PRSV and to PLDMV (Id.). In particular, two lines (numbers 493 and 494) with the DNA construct pNP-YKT3'PLDMV showed resistance to PRSV strains of KE, TH, and YK, and to PLDMV (Id.). In addition, a transgenic papaya (line number 423) with DNA construct pNP-YKT5'Jap showed resistance to PRSV strains KE, YK, TH, and Jap (Id.). Thus, the approach described by the present patent application is enabling for conferring multiple viral resistance, e.g., for PRSV and for PLDMV (Id.).

Furthermore, the nucleotide sequences of many viral plant pathogens are known and available to the skilled scientist (Gonsalves Declaration ¶ 14). For example, a

single search request designating nucleotides for "viral plant protein" on the National Center for Biotechnology Information (NCBI) on-line search site generated a list of 288 nucleotide sequences for coat proteins of virus associated with plant pathogenesis that are available on the public (Id.). It is common for research scientists to access publicly available viral genomic sequence information, carry out a BLAST or other type of homology search on that nucleotide relative to a second sequence of interest, and identify a potentially useful nucleotide sequence from a desired source for a given objective (Id.). Thus, it would be well within the capabilities of a skilled scientist to isolate or synthesize a fragment of 110 nucleotides or more of a desired trait DNA from a viral source for use in a DNA construct. (Id.)

In addition, the present application teaches several types and sources of other useful trait DNAs (pg. 17, lines 7-33), DNA molecules that can serve as DNA silencer molecules (pg. 18, lines 21-31), promoters and other regulatory regions that function to allow expression of heterologous DNA in host cells (pg. 21, line 16 to pg. 23, line 8), vectors suitable for expression of the nucleic acids of my invention in host cells (pg. 20, lines 11-35), and expression vector-host systems useful with the nucleic acid molecules of the present application (pg. 21, lines 1-15).

There are a multitude of protocols known to the skilled scientist that teach how to identify and isolate viral DNA, prepare DNA constructs in plant expression vectors, and to transform plants with such expression vectors (Gonsalves Declaration ¶ 15). For example, there are references that specifically disclose methods to obtain and manipulate nucleic acid molecules from plant viruses (Id.). Many other useful references are available which provide specific guidance to the skilled scientist for the transformation, regeneration, and testing of transgenic plants (Id.) For example, Gene Transfer to Plants, Potrykus and Spangenberg, eds., Springer Verlag Press, Berlin (1995), discloses methods for gene transfer to plants using *Agrobacterium*-mediated gene transfer (Parts I and II), protoplast transfer (Part III), biolistic transformation (Part IV), microinjection and fiber-mediated transformation (Part V), and tissue electroporation (Part VI), as well as methods for the analysis of transgenic plants post-transformation (Part VII), the establishment and maintenance of embryogenic cultures (Part VIII), and the use of genetic markers and expression signals in plant transformation (Part IX) (Id.).

Therefore, it is clear that a skilled scientist having read the present patent application would know how to make additional DNA constructs having a fragment of a trait

DNA from a viral source that has at least 110 nucleotides but is less than a full-length DNA, and a silencer DNA, under the control of a single promoter sequence and single terminator sequence, and how to use such constructs to prepare expression vectors and host cells, including plant cells, containing those nucleic acid molecule constructs, and, finally, how to prepare transgenic plants to impart a desired trait to the transformed plants (Gonsalves Declaration ¶ 16).

Therefore, applicants submit that the present invention as filed is fully enabling and that the rejection of claims 1-5, 9-12, 16-20, 23-25, 27-29, 31-36, 40-43, 46-50, 53-62, 66-74, 77-81, and 93-94 under 35 U.S.C. § 112 (1st para.) for lack of enablement should be withdrawn.

The rejection of claims 1-5, 9-12, 16-20, 23-25, 27-29, 31-36, 40-43, 46-50, 53-62, 70-74, 77-81, and 93-94 under 35 U.S.C. § 112 (2nd para.) for indefiniteness is respectfully traversed in view of the above amendments and as noted below.

The PTO has taken the position that the terms "trait DNA molecule" and "silencer DNA molecule" are indefinite. Applicants disagree.

As clearly stated in claim 1, as amended, the DNA construct includes a fragment of trait DNA from a viral source, having at least 110 nucleotides, but is less than a full-length cDNA encoding the trait. The term 'silencer DNA' was fully described in the present application as "virtually any nucleic acid" molecule (pg. 18, lines 21-23), and as claimed in the present invention is heterologous to the trait DNA and is heterologous to plants. Furthermore, the specification provides examples of the types of nucleic acid molecules that can be used as a silencer DNA molecule in the constructs of the present invention (pg. 18, lines 21-31; pg. 40, lines 33-39) as well as providing data demonstrating the efficacy of various silencer DNA molecules in conferring the trait of viral resistance to plants transformed with constructs of the present invention (Example 7). As the claims make clear, the fragment of a trait DNA molecule and the silencer DNA molecule are separate, required elements of the claims. The nature of these components is also readily apparent from the claims. Applicants, therefore, submit that the terms "trait DNA" and "silencer DNA" as described and used in the present application would be clearly understood by one of ordinary skill in the art.

Accordingly, the rejection of claims 1-5, 9-12, 16-20, 23-25, 27-29, 31-36, 40-43, 46-50, 53-62, 70-74, 77-81, and 93-94 under 35 U.S.C. § 112 (2nd para.) for indefiniteness is improper and should be withdrawn.

The rejection of claims 1-5, 9-12, 16-17, 19-20, 23-25, 27-29, 31-36, 40-41, 43, 46-50, 53-54, and 56-57 under 35 U.S.C. § 102(b) as anticipated by Lawson et al., "Engineering Resistance to Mixed Virus Infection in a Commercial Potato Cultivar: Resistance to Potato Virus X and Potato Virus Y in Transgenic Russet Burbank," Bio/Technol. 8: 127-34 (1990)("Lawson") is respectfully traversed.

Lawson teaches transforming a plant with the coat protein genes of the potato virus X ("PVX") and potato virus Y ("PVY) to produce transgenic plants that expressed both CP genes and that were resistant to infection by PVX and PVY. The expression vectors of Lawson having the PVY- or PVX-encoding DNA molecules contain the full length DNA molecule encoding PVY or PVX resistance, respectively (see pg. 128, right col., first full para.) and each DNA molecule is sufficient to independently impart a desired trait (i.e., PVX or PVY resistance) to plants transformed with the DNA molecule (see pg. 130, para. bridging col. 1 and 2 and the first full para. of col. 2). Thus, Lawson does not teach "a fragment of a trait DNA molecule which has a length that is insufficient to independently impart a desired trait to plants transformed with said fragment of a trait DNA molecule, wherein the fragment of trait DNA molecule is from a viral source and is at least 110 nucleotides in length but is less than a full-length cDNA encoding said trait". In addition, when the coat protein encoding DNA molecules are put in the same expression vector, they each remain under the control of a separate promoter (see Figure 2). Therefore, Lawson does not teach a DNA construct having "a single promoter sequence which effects transcription of both the fragment of a trait DNA molecule and the silencer DNA molecule", as in the claimed invention. Accordingly, Lawson cannot anticipate the claimed invention, and the rejection of claims 1-5, 9-12, 16-17, 19-20, 23-25, 27-29, 31-36, 40-41, 43, 46-50, 53-54, and 56-57 based on Lawson should be withdrawn.

The rejection of claims 1-4, 9-11, 16-17, 19-20, 23-25, 27-29, 31-35, 40-41, 43, 46-49, 53-61, 66-67, 69-74, 77-78, 80-81, and 93-94 are rejected under 35 U.S.C. 102(a) as anticipated by WO 96/21031 to Tricoli et al. ("Tricoli") is respectfully traversed.

Tricoli teaches a chimeric recombinant DNA molecule comprising a plurality of DNA sequences, each of which has a plant-functional promoter linked to a coding region which encodes a viral coat protein. The viral DNA sequences, each with its own promoter, are linked in tandem so that they are expressed in virus-susceptible plant cells transformed with the chimeric recombinant DNA molecule to impart resistance to the viruses.

The PTO has apparently interpreted Tricoli as preparing plant expression constructs having more then one viral coat protein-encoding DNA under the control of a single promoter. The PTO's interpretation appears to be based on the preparation by Tricoli of a plant expressible fusion gene consisting of the WMV2 coat protein and the NH_3-terminal portion of the cucumber mosaic virus ("CMV") coat protein gene (pg. 22, lines 15-28). However, the NH_3-terminal portion of the CMV gene used by Tricoli is a 70 nucleotide untranslated region of the CMV gene, which Tricoli uses in plant expression cassettes as an enhancer for translation, i.e., a 5' regulatory region. This is clearly described in Slightom, J.L., "Custom Polymerase-Chain Reaction Engineering of a Plant Expression Vector," Gene 100:251-255, (1991) ("Slightom") (at Abstract; pg. 251; and 252, 1st full para.) (attached hereto as Appendix B), which is cited by Tricoli at pg. 22, lines 23-24. The structure and function of the CMV-CP DNA segment in the construct is also made clear in Tricoli at pg. 23, lines 21-26. The CMV-CP/WMV2 construct is then further modified by the addition of the CaMV 35S plant promoter and polyadenylation signal to produce a plant expressible coat protein cassette, as described in Slightom (Tricoli at pg. 22, lines 20-24). Tricoli teaches that the expression cassettes for zucchini yellow mosaic virus ("ZYMV"), cucumber mosaic virus ("CMV"), and squash mosaic virus ("SQMV") coat protein are all prepared in the manner taught by Slightom. Each of the coat protein expression constructs thus prepared is defined by Tricoli as a single coat protein cassette (see pg. 24, line 11). To make multiple coat protein-expressing constructs, Tricoli takes single coat protein cassettes, each containing one full-length cDNA (i.e., an ORF) for a viral coat protein, coupled to the 70 nucleotide 5' untranslated region of the CMV Cp, a CaMV 35S promoter, and 35S terminator, and places the cassettes together in various combinations in a vector in order to obtain binary plasmids (see pg. 24, line 10 to pg. 26, line 9). Thus, Tricoli does not teach "[a] DNA construct comprising: a fragment of a trait DNA molecule which has a length that is insufficient to independently impart a desired trait to plants transformed with said fragment of a trait DNA molecule, wherein the fragment of a trait DNA molecule is from a viral source and is at least 110 nucleotides in length but is less than a full-length cDNA encoding said trait; a silencer DNA molecule effective to achieve post-transcriptional gene silencing of said fragment of a trait DNA molecule and coupled to said fragment of a trait DNA molecule," and that has "a single promoter sequence which effects transcription of both the fragment of a trait DNA molecule and the silencer DNA molecule; and a single termination sequence which ends transcription of both the trait DNA molecule and the silencer DNA molecule."

Therefore, Tricoli cannot anticipate the claimed invention as amended, and the rejection of claims 1-4, 9-11, 16-17, 19-20, 23-25, 27-29, 31-35, 40-41, 43, 46-49, 53-61, 66-67, 69-74, 77-78, 80-81 and 93 under 35 U.S.C. §102(a) over Tricoli should be withdrawn.

The rejection of claims 1-5, 9-12, 16-20, 23-25, 27-29, 31-36, 40-43, 46-50, 53-62, 66-74, 77-81, and 93-94 under 35 U.S.C. §103(a) for obviousness over Tricoli is respectfully traversed.

As discussed above, Tricoli does not teach the claimed DNA construct of the present invention or the use of such a construct to obtain plants with resistance to multiple viruses. It is the PTO's position that it would have been obvious to add an additional trait DNA molecule to the construct taught by Tricoli, in view of Tricoli's success in obtaining plants with resistance to multiple viruses, and thus obtain the DNA construct of the present application. Applicants respectfully disagree.

Tricoli does not teach or suggest a DNA construct having a DNA silencer molecule, let alone its use to impart a trait to transgenic plants where the fragment of a trait DNA molecule has a length which alone is insufficient to independently impart that trait. Furthermore, as described above, the expression vectors of Tricoli are constructed so that each viral protein is a full length DNA molecule encoding the viral CP of choice, and each viral CP DNA molecule is under the control of a promoter and a termination sequence. There is no suggestion or motivation in Tricoli to place multiple trait molecules under the control of a single promoter and terminator, as in the claimed invention. Furthermore, there is no suggestion in Tricoli that anything less than a full-length DNA encoding the desired trait would be effective to confer resistance to a plant transformed with such a construct. In contrast, the DNA construct of the present invention includes DNA molecules that include "a fragment of a trait DNA molecule," where the fragment is "at least 110 nucleotides in length but is less than a full-length cDNA encoding said trait" and has "a single promoter sequence which effects transcription of both the fragment of a trait DNA molecule and the silencer DNA molecule; and a single termination sequence which ends transcription of both the fragment of a trait DNA molecule and the silencer DNA molecule." Therefore, even if one had added additional DNA molecules to the construct of Tricoli, it would not have resulted in the DNA construct of the present invention.

Therefore, applicants submit that the rejection under 35 U.S.C. §103(a) for obviousness over Tricoli is improper and should be withdrawn.

In view of all of the foregoing, applicants submit that this case is in condition for allowance and such allowance is earnestly solicited.

Respectfully submitted,

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Date	Jo Ann Whalen
6/2/03	Jo Ann Whalen

Appendix A

Version With Markings to Show Changes Made

In reference to the amendments made herein to claims 1, 2, 20, 24, 28, 33, 47, 57, 59, 71, and 81, additions appear as underlined text, while deletions appear as bracketed text, as indicated below:

In The Claims:

1. (Three Times Amended) A DNA construct comprising:

a fragment of a trait DNA molecule which has a length that is insufficient to independently impart a desired trait to plants transformed with said fragment of a trait DNA molecule, wherein the fragment of a trait DNA molecule is from a viral source and is at least 110 nucleotides in length but is less than a full-length cDNA encoding said trait;

[and] a silencer DNA molecule effective to achieve post-transcriptional gene silencing of said fragment of a trait DNA molecule and coupled to said fragment of a trait DNA molecule, wherein said fragment of a trait DNA molecule and said silencer DNA molecule are heterologous to each other and collectively impart the trait to plants transformed with said DNA construct and wherein said fragment of a trait DNA molecule and said silencer DNA molecule are heterologous to plants;

a single promoter sequence which effects transcription of both the fragment of a trait DNA molecule and the silencer DNA molecule; and

a single termination sequence which ends transcription of both the fragment of a trait DNA molecule and the silencer DNA molecule.

2. (Twice Amended) The DNA construct according to claim 1, wherein said DNA construct comprises:

a plurality of different trait DNA molecules[, at least one of which has a length that is insufficient to impart the different trait to plants transformed with that different trait DNA molecule] operatively positioned within said DNA construct so that said single promoter sequence and said single termination sequence, respectively, effect transcription and end transcription of said plurality of different trait DNA molecules.

20. (Twice Amended) The DNA construct according to claim 1, wherein said construct effects post-transcriptional gene silencing of the fragment of trait DNA molecule within plants.

24. (Twice Amended) The DNA expression vector according to claim 23, wherein said DNA construct comprises a plurality of different trait DNA molecules[, at least one of which has a length that is insufficient to impart the different trait to plants transformed with that different trait DNA molecule] operatively positioned within said DNA construct so that said single promoter sequence and said single termination sequence, respectively, effect transcription and end transcription of said plurality of different trait DNA molecules.

28. (Twice Amended) The host cell according to claim 27, wherein said DNA construct comprises a plurality of different trait DNA molecules[, at least one of which has a length that is insufficient to impart the different trait to plants transformed that different trait DNA molecule] operatively positioned within said DNA construct so that said single promoter sequence and said single termination sequence, respectively, effect transcription and end transcription of said plurality of different trait DNA molecules.

33. (Twice Amended) The transgenic plant according to claim 32, wherein said DNA construct comprises a plurality of different trait DNA molecules[, at least one of which has a length that is insufficient to impart the different trait to plants transformed with that different trait DNA molecule] operatively positioned within said DNA construct so that said single promoter sequence and said single termination sequence, respectively, effect transcription and end transcription of said plurality of different trait DNA molecules.

47. (Twice Amended) The method according to claim 46, wherein said DNA construct comprises a plurality of different trait DNA molecules[, at least one of which have a length that is insufficient to impart the different trait to plants transformed with that different trait DNA molecule] operatively positioned within said DNA construct so that said

Appendix A

Version With Markings to Show Changes Made

<u>single promoter sequence and said single termination sequence, respectively, effect transcription and end transcription of said plurality of different trait DNA molecules</u>.

57. (Twice Amended) The method according to claim 47 further comprising:

propagating progeny of the [transgenic] <u>plants transformed with said DNA construct</u>.

59. (Twice Amended) The transgenic plant seed according to claim 58, wherein said DNA construct comprises a plurality of different trait DNA molecules[, at least one of which has a length that is insufficient to impart that different trait to plants transformed with that different trait DNA molecule] <u>operatively positioned within said DNA construct so that said single promoter sequence and said single termination sequence, respectively, effect transcription and end transcription of said plurality of different trait DNA molecules</u>.

71. (Twice Amended) The method according to claim 70, wherein said DNA construct comprises a plurality of different trait DNA molecules[, at least one of which have a length that is insufficient to impart that different trait to plants transformed with that different trait DNA molecule] <u>operatively positioned within said DNA construct so that said single promoter sequence and said single termination sequence, respectively, effect transcription and end transcription of said plurality of different trait DNA molecules</u>.

81. (Twice Amended) The method according to claim 71 further comprising:

propagating progeny of the [transgenic] plant<u>s transformed with said DNA construct</u>.

APPENDIX B

Gene, 100 (1991) 251–255

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GENE 03961

Custom polymerase-chain-reaction engineering of a plant expression vector

(Recombinant DNA; cauliflower mosaic virus 35S gene-regulatory elements; 5′-untranslated region; viral coat protein-encoding genes; PCR; CPCR)

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SUMMARY

Polymerase-chain-reaction (PCR) amplification combined with custom-synthesized oligodeoxyribonucleotide (oligo) primers can be used to make complex genetic engineering steps (e.g., translational fusions) easy. Much of the complexity of the engineering steps can be incorporated into the custom oligo primers. Using this technique, a plant constitutive expression vector, pUC18cpexp, was constructed. This vector is based on the cauliflower mosaic virus 35S gene-regulatory elements and the cucumber mosaic virus coat protein-encoding gene (*cp*) 5′-untranslated region. Use of this vector is demonstrated by modifying the *cp* genes of several plant viruses and cloning them into pUC18cpexp. Because the construction and use of this vector system require custom oligo primer synthesis and PCR amplification, the technique is referred to as custom PCR engineering.

INTRODUCTION

The PCR amplification technique, using the thermostable *Taq* polymerase (Saiki et al., 1988), has many uses in molecular biology procedures, which include molecular cloning, nt sequencing, and sequence modifications (Innis et al., 1989). A unique feature of PCR is its ability to amplify discrete regions of DNA and to place useful restriction enzyme sites adjacent to the amplified region. In this report the engineering of the plant expression vector pUC18cpexp using CPCR amplification is described. This vector contains the regulatory elements of the CaMV 35S gene (Pietrzak et al., 1986) and most of the CMV 5′-untranslated region (Quemada et al., 1989). Several viral 5′-untranslated regions have been shown to be translational enhancers in plant expression systems (Gallie et al., 1987; Jobling and Gehrke, 1987). In this example, the 5′-untranslated region of the CMV *cp* gene was selected because of its availability; it appears to also serve as a translational enhancer (Quemada et al., 1991). An *Nco*I cloning site was placed at the junction of the CMV-translation start codon (ATG) and 35S terminator DNA region. Even though this expression cassette contains only the *Nco*I site for cloning, its use can be greatly extended if the targeted coding regions are also PCR-amplified to add flanking *Nco*I sites. MacFerrin et al. (1990) have reported a similar use of PCR engineering for the construction of *Escherichia coli* expression vectors.

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Abbreviations: bp, base pair(s); CaMV, cauliflower mosaic virus; CMV, cucumber mosaic virus; CP, coat protein; *cp*, gene (DNA) encoding CP; CPCR, custom PCR; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; PCR, polymerase chain reaction; PRV, papaya ringspot virus; SV40, simian virus 40; WMV, watermelon mosaic virus; ZYMV, zucchini yellow mosaic virus.

EXPERIMENTAL AND DISCUSSION

(a) CPCR construction f pUC18cpexp

The *cp* gene of CMV was engineered for expression in plants by cloning it between the regulatory sequences of the CaMV 35S gene (Quemada et al., 1991). The engineering of the CMV *cp* gene was straightforward because it contains a 5′-untranslated region (70 nt) and a translation start codon (ATG) (Quemada et al., 1989). Thus it was cloned (using convenient restriction enzyme sites) between the regulatory elements of the CaMV 35S gene to obtain the clone pUC1813cpCMV19 (Fig. 1). However, the *cp* genes of the potyvirus group of plant viruses (which include PRV, WMVII and ZYMV) are much more difficult to engineer because their proteins are encoded within a long single polypeptide (see review by Dougherty and Carrington, 1988). The individual proteins are cleaved from the polyprotein by proteases that are part of the polyprotein. Thus, for the expression of a potyvirus *cp* gene in plants both the important transcriptional and translational elements must be added.

The CMV *cp* gene expression vector pUC1813cpCMV19 contains all the regulatory elements needed for plant expression, but it cannot be conveniently used for the expression of potyvirus *cp* genes because each construct would require a different translational fusion. However, by using CPCR amplification of the vector pUC1813cpCMV19 a more useful plant expression vector, pUC18cpexp, was constructed that allows translational fusions to be made at the site of the translation start codon (ATG). The restriction enzyme recognition site for *Nco*I (CCATGG) was selected because it shares considerable identity with the plant translation consensus sequence (AACAATGGC) described by Lutcke et al. (1987).

CPCR amplifications of the regulatory elements of pUC1813cpCMV19 were done using the oligo primer sets shown in Fig. 1. The oligo primers JLS-81 and -82 (Fig. 1) amplified a 400-bp region that contains 330 bp of the CaMV 35S promoter and the 70-bp CMV 5′-untranslated region. The oligo primers JLS-83 and -84 (Fig. 1) amplified a 200-bp region that contains CaMV 35S terminator region, including the poly(A)-addition signal. These oligo primers added *Hin*dIII sites to the 5′ and 3′ ends of these CaMV 35S promoter and terminator fragments and *Nco*I sites to the 3′ and 5′ ends of these fragments, respectively (Fig. 1). CPCR amplification yielded the expected 400-bp promoter (*P*) and 200-bp terminator (*T*) fragments shown in Fig. 2. These fragments were removed from the gel matrix, digested with *Nco*I, and ligated together to obtain the combined 600-bp fragment (*PT* in Fig. 2). This 600-bp fragment was amplified a second time using oligo primers JLS-81 and -84 (Fig. 1) to increase its concentration. The double-amplification 600-bp fragments were digested with *Hin*dIII, polyacrylamide gel purified, and ligated into *Hin*dIII-cut pUC18. After transforming *E. coli* cells five clones were selected for nt sequencing to check for ligation and/or PCR-generated mutations. This analysis showed that these clones contained identical inserts and no nt sequence mutations. This nt sequence analysis (a total of about 3000 bp from these five clones) was encouraging as it indicates that

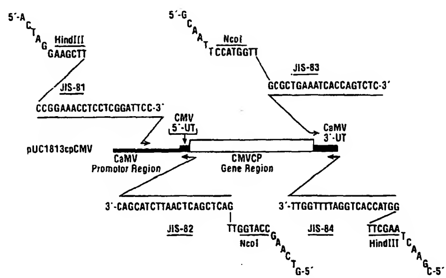

Fig. 1. Strategy used to CPCR-engineer the plant expression vector pUC18cpexp. The gene structure of clone pUC1813cpCMV19 (used for expressing the CMV *cp* gene; Quemada et al., 1991), the location and sequence of the four oligo primers used for CPCR are shown. The restriction site synthesized within each oligo primer, internal *Nco*I and flanking *Hin*dIII sites are also indicated (overlined or underlined). Each oligo primer contains an additional six nt adjacent to the restriction enzyme site (written on the diagonal) to aid restriction enzyme binding and DNA cleavage. Shared nt sequences between the oligo primers and the amplified regions are denoted by a line above or below the oligo sequence.

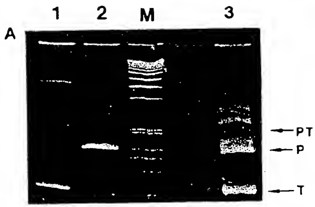

Fig. 2. CPCR amplification results for the promoter and terminator regions used to construct pUC18cpexp. The amplification of 200-bp CaMV terminator (lane 1) and 400-bp CaMV-CMV promoter (lane 2) regions are shown. Lane 3 shows the results of ligating these *Nco*I-cut 200-bp and 400-bp fragments to obtain the 600-bp fragment (labeled *PT*); many fragments did not ligate as indicated by the intensity of the 200-bp terminator fragment (labeled *T*) and 400-bp promoter fragment (labeled *P*). **Methods:** CPCR amplification reactions contained 50 ng of each primer and 100 ng of template (pUC1818cpCMV19) in a total reaction volume of 100 μl. Buffer and nt concentrations are as described by the vendor, Perkin-Elmer-Cetus. Reaction mixtures were subjected to 30 cycles of amplification using a denaturation step of 94°C (2 min), annealing step at 50°C (1 min), and extension step of 74°C (3 min). DNA amplification cycles were controlled by a programmable cyclic reactor obtained from Ericomp, Inc. After amplification, samples were extracted once with a phenol–chloroform–isoamyl alcohol mixture (1 : 1 : 0.04), once with a chloroform–isoamyl alcohol mixture (1 : 0.02), precipitated with ethanol, and then resuspended in 30 μl of H_2O. Samples were then subjected to digest by *Nco*I followed by electrophoreses through a 7% polyacrylamide–15% glycerol gel. CPCR amplified bands were visualized by ethidium bromide staining and the DNA fragments were removed from the gel matrix by electroelution (Maniatis et al., 1982). These fragments were combined in equal concentration (about 0.5 μg each), then 10 units of T4 ligase (Collaborative Research, Inc.) were added and the volume adjusted to a total of 10 μl. The ligation reaction was incubated at 14°C for 18 h, after which the ligase was inactivated by heating to 65°C for ten min, then loaded on a polyacrylamide-glycerol gel. Ligation products show the presence of the expected 600-bp promoter and terminator fragment (labeled *PT*) and other products that resulted from the other possible ligation events, i.e., the 400-bp fragment (ligation of two terminator fragments) and the 800-bp (ligation of two promoter fragments).

PCR-generated errors did not occur at a frequency that would render the CPCR engineering strategy useless. The nt sequence of the CaMV-CMV regulatory regions of the CPCR-engineered expression cassette pUC18cpexp are included in Fig. 3.

(b) Cloning PRV, WMVII, and ZYMV *cp* genes into pUC18cpexp

CPCR engineering strategies were designed for the cloning of the PRV, WMVII and ZYMV *cp* genes into pUC18cpexp. These strategies were similar and straightforward as CPCR amplifications were used to add the necessary *Nco*I sites flanking each *cp* gene. The goal was to obtain a translational fusion that would upon expression produce a CP as identical as possible to the respective native CP. Some modifications were unavoidable due to the addition of an N-terminal methionine residue and because the *cp* translation fusion codon selection required conserving the *Nco*I recognition site (CCATGG). Thus the translation start codon was generally fused to the first *cp* codon beginning with a G nt. In addition, since it appeared that the preferred N-terminal aa for potyvirus CP might be Ser, Gly, or Ala (Dougherty et al., 1989), these codons were preferentially selected. Addition of the *Nco*I site to the 3′ end of these *cp* genes was much simpler because these linkages only involved transcriptional fusions.

Oligo primers designed for CPCR amplification of the PRV *cp* gene fused the translation start codon to the fifth aa (Ala) that follows the expected Glu-Ser peptide cleavage site (Quemada et al., 1990a). The 3′-*Nco*I site was added 50 bp downstream from the PRV translation stop codon (Fig. 3). CPCR amplification of the plasmid pPRV117 (Quemada et al., 1990a) yielded a 900-bp fragment that was digested with *Nco*I and then cloned into the *Nco*I-digested vector pUC18cpexp. Two selected clones were completely sequenced and no mutations were found (a total of 1800 bp were checked). Fig. 3 shows the nt sequence of CaMV-CMV regulatory elements of pUC18cpexp and its junctions with the PRV *cp* gene in the clone referred to as pUC18cpPRV.

Similar CPCR amplifications were used for the engineering of the *cp* genes from WMVII and ZYMV (data not shown). Oligo primers designed for CPCR amplification of the WMVII *cp* gene fused the translation start codon to the second aa codon, Gly, of the *cp* gene (Quemada et al., 1990b). CPCR amplification of the clone pWMVII-3.2 (Quemada et al., 1990b) yielded a 900-bp fragment that was cloned into pUC18cpexp and the clone designated pUC18cpWMVII was isolated and sequenced (data not shown), no PCR and/or ligation-generated mutations were found. CPCR amplification of the ZYMV *cp* gene fused the translation start site to the second aa, Gly, of the *cp* gene (Quemada et al., 1990b). CPCR amplification of clone pZYMV-15 (Quemada et al., 1990b) yielded a 900-bp fragment that was cloned into pUC18cpexp and the clone referred to as pUC18cpZYMV was selected and sequenced. This sequence revealed a cloning artifact at the *Nco*I site near the translation start site, it contained only one C nt instead of the expected CC nt (data not shown). The nt sequence of the remaining ZYMV *cp* gene revealed no additional errors.

(c) Conclusions

PCR amplification is a powerful method for the modification of DNAs and it was used here for the construction of

```
       HindIII
       ------
       GAAGCTTCCGGAAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTTATTGTGAAG
   1   ------------------------------------------------------------   60

       ATAGTGGAAAAGGAAGGTGGCTCCTACAAATGCCATCATTGCGATAAAGGAAAGGCCATC
  61   ------------------------------------------------------------  120

       GTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATC
 121   ------------------------------------------------------------  180
                                                             <------
       GTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCC
 181   ------------------------------------------------------------  240
       ----------------CaMV Promoter-------------------------------
       ACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCTATATAA
 241   ------------------------------------------------------------  300
       --------------------------------->
                                         <--------------------------
       GGAAGTTCATTTCATTTGGAGAGGACACGGTACCCCTACTGACTATATAGAGAGTGTGTG
 301   ------------------------------------------------------------  360
       ---CMV 5'-Untranslated Region---------------->
                                                    NcoI
                                                   ------
                                  JLS-86   ATCATTCCATGG
                                                       ----------
       TGCTGTGTTTTCTCTTTTGTGTCGTAGAATTGAGTCGAGTCAACCATGGCTGTGGATGCT
 361   ------------------------------------------------------------  420
                                                      MetAlaValAspAla
       ------
       GGTTTGAATGAAAAACTCAAAGAGAAGGAAAATCAGAAAGAAAAAGAAAAAGAAAAACAA
 421   ------------------------------------------------------------  480
       GlyLeuAsnGluLysLeuLysGluLysGluAsnGlnLysGluLysGluLysGluLysGln

                         [  PRV Coat Protein Gene  ]

       AGACACACACAGTGGAAGATGTCAATAGAGACATGCACTCTCTCCTGGGTATGCGCAACTAA
1201   ------------------------------------------------------------  1260
       ArgHisThrValGluAspValAsnArgAspMetHisSerLeuLeuGlyMetArgAsnEnd
                                                        NcoI
                                                       ------
       ATACCTGCGCTTGTGTGTTTGTTGAGTCTGACTCGACCCTGTTTCACCCCATGGTTGCGC
1261   ------------------------------------------------------------  1320
                                GACTGAGCTGGGACAAAGTGGGGTACC
                                                          AATCGA   JLS-86
       TGAAATCACCAGTCTCTCTCTACAAATCTATCTCTCTCTATAATAATGTGTGAGTAGTTC
1321   ------------------------------------------------------------  1380

       CCAGATAAGGGAATTAGGGTTCTTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAAC
1381   ------------------------------------------------------------  1440

       CCTTAGTATGTATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATTCCTAAAA
1441   ------------------------------------------------------------  1500
                         HindIII
                         ------
       CCAAAATCCAGTGGTACCAAGCTTC
1501   -------------------------  1525
```

Fig. 3. The nt sequence of CPCR-engineered expression vector pUC18cpPRV. The nt sequence was obtained using double-stranded DNA (Zagursky et al., 1985) and the dideoxy sequence enzymatic sequencing method (Sequenase™2, U.S. Biochemicals). The DNA sequencing gel system used has been described by Slightom et al. (1991) and employs 1-meter gels; sequence readings in the range of 800 bp from the oligo primer are routinely obtained. The location of the CaMV promoter and terminator and CMV 5′-untranslated regions is shown. Also shown are the nt sequence and location of the oligo primers used for CPCR-amplification of the PRV *cp* gene. The location of the *Nco*I sites used for cloning the CPCR amplified PRV *cp* gene into pUC18cpexp is shown. Shared sequences between the oligo primers and PRV *cp* gene are shown as dashed lines above the sequence line and as the complementary nt sequence below the sequence line. The complete PRV *cp* gene sequence is not shown as its sequence has previously been described (Quemada et al., 1990a).

a CaMV-based plant-expression cassette. The major strength of this method is that it allows complex DNA modification to be done by oligo primer synthesis followed by PCR amplification and straightforward ligation events. A major weakness of the strategy is the concern that *Taq* polymerase lacks fidelity. However, analysis of the fidelity

of *Taq* polymerase finds it to be similar to that found for other polymerases, T7 and DNA polymerase I (Keohavong and Thilly, 1989). In addition, the probability of obtaining PCR-generated errors in the examples presented here are reduced because the initial template is cloned and its concentration is not limiting, as is the case when amplifying rare mRNAs or single-copy genomic DNAs. This is supported by nt sequence analyses of the CPCR-engineered clones described here (a total of 8400 bp) since no PCR-generated errors were found. However, it was surprising to find an error that resulted from a ligation event; thus, it is recommended that CPCR-engineered DNAs be sequenced to ensure accuracy.

The constitutive plant expression vector pUC18cpexp can be effectively used for the cloning of almost any coding region provided that it is modified by CPCR amplification. However, coding regions that contain multiple *Nco*I sites will be problematic and may require the selection of a different restriction enzyme site to be placed at or near the translation start codon. Each of the potyvirus *cp* expression vectors described here has been transferred into plant tissues and found to be functional (S. Namba, K. Ling, C. Gonsalves, J.L.S. and D. Gonsalves, manuscript in preparation).

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